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DOI:

[10.1002/bit.25915](https://doi.org/10.1002/bit.25915)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Jamshidi, P, Chouhan, G, Williams, R, Cox, SC & Grover, LM 2016, 'Modification of gellan gum with nanocrystalline hydroxyapatite facilitates cell expansion and spontaneous osteogenesis', *Biotechnology and Bioengineering*. <https://doi.org/10.1002/bit.25915>

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Checked April 2016

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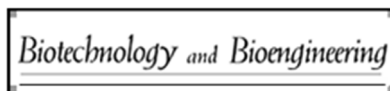
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Journal:	<i>Biotechnology and Bioengineering</i>
Manuscript ID	15-808.R1
Wiley - Manuscript type:	Article
Date Submitted by the Author:	02-Nov-2015
Complete List of Authors:	Jamshidi, Parastoo; University of Birmingham, school of Metallurgy and Material Chouhan, Gurpreet; University of Birmingham, School of Chemical Engineering Williams, Richard; University of Birmingham, School of Chemical Engineering Cox, Sophie; University of Birmingham, School of Chemical Engineering Grover, Liam; University of Birmingham, School of Chemical Engineering
Key Words:	Gellan gum-hydroxyapatite, Nano-composite, cell attachment, spinner flask, differentiation, osteogenesis

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Modification of gellan gum with nanocrystalline hydroxyapatite facilitates cell expansion and spontaneous osteogenesis

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Running title: The loading of nanocrystalline hydroxyapatite into gellan gum stimulates osteogenic differentiation

Abstract

Nanocomposites composed of hydrogels and calcium phosphates are of great interest in the development of bone graft replacements since they may have a structural and compositional resemblance to bone. Culture beads formed from such materials could be used in stirred tank culture and thereby enable cell expansion in a sufficiently efficient manner to allow for the generation of enough large number of cells for large-scale bone reconstruction. Although combinations of materials such as alginate, collagens and various calcium phosphates have been investigated as culture beads, these materials are unsuitable for application since they have been shown to rapidly degrade in physiological conditions and enable relatively little tailoring of mechanical properties. In this study, gellan gum-nano sized hydroxyapatite (nHA) composites, which have been shown to be resistant to degradation and easily modified with respect to modulus, were formulated and characterised as regards their ability to enable cell attachment and proliferation. It was shown that the addition of 5wt% of nHA to the culture beads enabled cell attachment and that an increase in nHA concentration to up to 25wt% enhanced the rate of cell proliferation. Most importantly, it was demonstrated that the addition of nHA to the cell culture beads enabled the formation of nodules in culture of MC3T3-E1 cells and strikingly stimulated the osteogenic differentiation of bone marrow stromal cells in the absence of osteogenic media when compared with tissue culture plastic (TCP) with the same condition.

Keyword: Gellan gum-hydroxyapatite, Nano-composite, cell attachment, spinner flask, differentiation, osteogenesis

Introduction

Although many materials exist that enable regeneration of bone (Stevens 2008, Chen 2013) to date no-one has developed a method that adequately enables the large scale regeneration of bone (Srour, Fogel et al. 2015). At present this requires the use of large-scale metallic fixation and autograft tissues to allow some level of bone in-growth to occur (Cancedda, Dozin et al. 2003). The limited volume of available autograft tissue means that allograft or xenograft (Shin, Yoshimoto et al. 2004) tissues must be employed to make up any shortfall in bone volume and the removal of vital components (Zhang, Powers Jr et al. 1997, Finkemeier 2002) to mediate osteogenesis during tissue processing can reduce the overall success of the procedures. Furthermore, poor revascularisation of the tissue graft results in a long-term chance of graft failure (Kaveh, Ibrahim et al. 2010). Cell therapy represents one way to mitigate the problems associated with the supply of tissue grafts (Cancedda, Dozin et al. 2003) and offers the tantalising opportunity of producing large volumes of well vascularised tissue following application. There is now a significant body of work that focusses on the use of cells that are derived from the bone marrow or periosteum (marrow stromal cells or mesenchymal stem cells) to mediate the regeneration of tissues, particularly hard tissues (Wakitani, Imoto et al. 2002, Caplan 2005). Despite this, however, to date there have been no significant clinical advances using these technologies. The major barriers to the ultimate translation of the technologies are 1) an insufficient number of cells for an effective therapy (Boo, Selvaratnam et al. 2011) and 2) phenotypic drift of cells that are adhered to the surface of the culture beads that are used to facilitate cell expansion (Çetinkaya, Kahraman et al. 2011, Chen, Reuveny et al. 2013).

Micro-carrier-based culture of adhesion dependent cells is one method that enables the expansion of cell number in a sufficiently efficient manner that it is possible to generate enough cells for single, large-scale therapies (Jin, Kim et al. 2012, Chen, Reuveny et al.

2013). At present, however, this process is mostly undertaken using commercially available cell culture beads that are typically formed from dextran or another material that is not suitable for application in a human (Sautier, Nefussi et al. 1992, Baker and Goodwin 1997, Malda, Van Blitterswijk et al. 2003). As a consequence, there is a requirement that the cells are stripped from the surface to which they adhere before being implanted, which adds a processing step and can also change the behaviour of the adherent cell population (Park, Pérez et al. 2013). Therefore, there is a major need for the development of micro-carriers formed from materials that are suitable for implantation to reduce the number of steps required during the manufacturing process. Such materials should have precisely defined properties, such as modulus, since these factors are well known to result in cell differentiation (Malda and Frondoza 2006; Park et al. 2013a). Using these structures in an appropriate cell culture system allows for the accurate control of process variables such as CO₂ concentration, pH value and local ionic concentrations (Malda and Frondoza 2006).

A class of materials with significant potential as cell culture beads are hydrogels, since they can be easily processed in ambient conditions to incorporate biological agents capable of controlling cell differentiation (Wang, Gong et al. 2008, (Anseth, Metters et al. 2002). At present, however, the practical application of these materials has been hindered by the fact that they do not readily enable the attachment of cells to their surface (Malafaya, Silva et al. 2007). While chemical modifications have been made to the surface of these structures to facilitate cell attachment (Lee and Mooney 2012), such innovations are yet to find their way into widely used therapeutic technologies. Another, simpler approach to facilitate the attachment of cells to the surface of the hydrogel materials is through the incorporation of a secondary phase that more readily facilitates cell attachment. To achieve this, a number of authors have modified hydrogel materials with calcium phosphate particles, which has to

some extent enabled better cell attachment (Lin and Yeh 2004, Rungsiyanont, Dhanesuan et al. 2011). Despite this, however, little is known of how these particles are likely to influence the mechanical properties that are exhibited by the material. Given that this is likely to direct differentiation, it is clearly a very important factor that needs investigating before application of the culture beads for the reproducible manufacture of cell therapies is possible. Our previous work has also demonstrated that the form of calcium phosphate particle that is used for the modification of the hydrogel matrix is highly dependent on particle diameter. We demonstrated that nanoparticulate hydroxyapatite (nHA) had a significantly more potent stiffening effect on the hydrogel in comparison with microparticles of the same composition (Jamshidi, Ma et al. 2012). In this study, we have evaluated the influence that incorporated nHA into gellan gum (GG) hydrogel in the form of culture beads can have on cell expansion and osteogenesis in vitro. GG has been used in medical formulations such as eye-drops due to being virtually endotoxin-free (Smith, Shelton et al. 2007, Kuno and Fujii 2011). Other recent studies also investigated the use of GG hydrogel for formation of a functional cartilage using this hydrogel in a cartilage augmentation device (Correia, Pereira et al. 2014).

The optimum level of incorporated nHA into GG matrix required to initiate cell attachment and the effect of increasing nHA loading on cell proliferation rate were investigated. The feasibility of using the GG/nHA beads in spinner flask cultures for cell expansion was also evaluated and compared with that of conventional monolayer cultures and static beads culture. Furthermore the ALP activity of the MC3T3-E1 pre-osteoblast cells grown on GG/nHA beads and also preliminary experiments with BMSC cells were performed to evaluate the ability of the fabricated beads to produce mineralised matrix and nodules in the presence or absence of osteogenic media.

Materials and methods

Synthesis of precursors and culture beads

Nano-scale HA particles were prepared by precipitation. Briefly, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (calcium nitrate, Fisher Scientific, Leicestershire, UK) and $(\text{NH}_4)_2\text{HPO}_4$ (ammonium phosphate, Fisher Scientific, Leicestershire, UK) were dissolved in double-distilled water. The pH of both suspensions was adjusted to 11 with concentrated NH_4OH (aqueous ammonia, Fisher Scientific, Leicestershire, UK). The $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ was added dropwise to the vigorously stirred $(\text{NH}_4)_2\text{HPO}_4$, whilst maintaining the pH at 11 by further addition of NH_4OH . The final suspension was then left to stir for 1 h at room temperature. This resulted in the formation of a milky white precipitate. The precipitated HA was separated from the solution by centrifugation at 3000 rpm for 5 min, which was repeated five times. The HA slurry was dried at 60°C for 24 h and the dried HA was then ground to powder using a pestle and mortar (Jamshidi, Ma et al. 2012).

GG/nHA beads with diameter of 415.75 ± 39.28 were prepared using a water-in-oil emulsion method. Briefly, the GG solution was prepared by dissolving low-acyl gellan powder in distilled water at 2.5% (w/w) at 90°C . HA sol was prepared by dispersing the nHA powder in distilled water with the homogenizer at 24,000 rpm for 3 min. The sol was then added into 2.5% gellan solution at 90°C . The mixture of GG/nHA solution was added into pre-heated 90°C oil phase under stirring at 500 rpm for 10 minutes. The mixture was finally transferred into an excess quantity of aqueous 1wt% CaCl_2 solution to enhance gel strength. GG beads were also prepared with the aforementioned method without addition of HA sol into the GG solution as a control. The fabricated beads were sterilized with ethanol (70%; Fisher Scientific) and then immersed in PBS and left overnight under ultraviolet light to complete the sterilization process prior to cell culture.

To determine the optimum concentration of nano-hydroxyapatite loaded into the GG matrix to facilitate cell attachment, various HA contents of 0.25%, 2.5%, 5% and 25% (w/w) were used to manufacture nHA/GG culture beads.

Disc-shaped samples of the GG/nHA composites were also prepared by pouring the GG/nHA mixture into a disk mould of diameter 13mm and thickness of 2mm and transferred to a refrigerator at 4°C to accelerate gel formation. Samples were then sterilized using the same method as for the beads.

Cell culture

Mouse MC3T3-E1 cells were used to evaluate the potential of GG/nHA beads as candidate culture beads for cell delivery. The MC3T3-E1 cell line was purchased from LGC (Middlesex, UK). The cells were grown in D-MEM (Dulbecco's Modified Eagle Medium) (Sigma, UK) which was supplemented with 10% FBS (PAA, Somerset, UK) 2.4% L-glutamine, 2.4% HEPES buffer, and 1% penicillin/ streptomycin. All cultures were maintained in sterile conditions at 37°C with 5% CO2 and 100% relative humidity and media was changed every two days.

The GG beads and GG/nHA beads were used in a concentration of 100 mg approximately of 200 beads per well of Sylgard pre-coated 24-well plate. The total surface area of the beads in each well was around 2.2cm² (equivalent to the surface area of a well of a 24-well plate as control). Cells were seeded onto the sterile GG, GG/nHA beads and disc-shaped samples separately at a final density of 2 x10⁴ cells /cm² on the surface of GG /nHA beads and GG /nHA disks in wells containing 2ml DMEM. At each time point, culture beads and disk-shapes samples were collected from the media then transferred to a new well-plate for

determination of cell attachment and proliferation using the MTT assay. Mitochondria of viable cells reduce the yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, which is dissolved by acidic isopropanol after removal of the culture medium. The absorbance of this colored solution can be quantified by measuring at a wavelength of 620 nm using a microplate reader (BIO-TEK, US), which gives an indication of cell number (Jamshidi, Bridson et al. 2013).

The number of adherent cells on to GG /nHA beads was also labelled with 40',6-diamidino-2-phenylindole (DAPI), a fluorescent dye and samples were visualised using confocal laser microscopy (Leica, UK).

Cell culture in spinner flasks

For stirred cultures, 25-ml spinner flasks (Wheaton Co., USA) with a final volume of 25 ml were used. The spinner flasks were placed on a magnetic stirrer inside a 37°C incubator with 5% CO₂. The GG/nHA beads were used in a concentration of 180 mg, per flask. The total available surface area of the beads in each flask was around 2.2 cm² (equivalent to the surface area in static condition). Cell seeding was performed at final density of 2 x10⁴ cells/cm² surface of nHA/GG beads with intermittent stirring for 2 minutes every 30 minutes at 25 rpm for 3 hours, it was then increased to 45 rpm for 1.5 hours. Continuous stirring at 45, 50, 55 to a final 65 rpm was continued at 15 minute intervals (Tebb et al. 2006). Sampling of the cell-bead complexes were performed at required time-points for evaluation of cell adherence and proliferation.

The cell adherence and proliferation was determined using MTT assay and scanning electron microscopy was used to observe cell morphology and distribution.

Scanning Electron Microscopy (SEM)

The cell-bead complexes were collected at required time-points, they were fixed in 1.5% glutaraldehyde (Sigma-Aldrich, UK) at 4°C overnight. Cells were then dehydrated through a series of increasing concentrations of ethanol (50, 70, 80, 90, and 100%) for 10 min at each concentration. This process then was continued by immersing in dry ethanol for 10 minutes. After dehydration, the cell-beads were critical-point-dried with CO₂, coated with gold and examined under a scanning electron microscope (SEM; quanta200, Fei, CZ).

Alkaline Phosphatase Activity (ALP)

The functional activity of the MC3T3-E1 pre-osteoblast cells grown on GG/HA beads was examined by measuring ALP activity. The alkaline phosphatase activity was assayed according to the method of Lowry et al. (Lowry, Rosebrough et al. 1951). Briefly, culture beads were collected from the media at various time intervals. The samples were washed with PBS and then suspended in 500µL PBS containing 100 mM glycine, 1 mM MgCl₂ and 0.05% Triton X-100 for 10 min. Aliquots of 60 µl were incubated with 300 µl of p-nitrophenyl phosphate solution at 37°C for 45 min. After adding 900 µl of ice cold 250 mM NaOH, the quantity of p-nitrophenol liberated was measured by monitoring absorbance at 405 nm using a UV-Vis plate reader.

Alizarin red staining

Furthermore mineralised matrix and nodules formation was estimated by Alizarin Red S staining, at interval times. Alizarin Red S, an anthraquinone derivative, forms as alizarin red-S-calcium in the chelation process with calcium existing in the mineralized matrix. This produces a birefringent staining. At each interval time, the cell-bead complexes were collected and fixed with 3.7% formaldehyde (Sigma-Aldrich, UK) in PBS for 20 min at room

temperature and washed with PBS. The complexes were stained in 40mM Alizarin Red S (Sigma Aldrich, UK) pH 4.2, for 5 min, and washed thoroughly five times with distilled water; the red matrix was visualised using light microscopy (Axiolab, Zeiss, Oberkochen, Germany).

The same experiment was performed with Primary rat bone marrow stromal cells (BMSCs) to demonstrate the potential of these beads to stimulate osteogenic differentiation. The BMSCs were extracted from Wistar rats following sacrifice. To isolate these cells, recently the femora were dissected from sacrificed adult albino Wistar rats. The soft tissue was cleaned from the femora using a scalpel and then they were placed into a transport medium that contained: Minimum Essential Medium (α -MEM ;), 10% penicillin/streptomycin, 2.5% HEPES, and 1% amphotericin (all from Sigma Aldrich, UK) until required. Subsequently, the epiphyses were removed and the femora were washed using supplemented α -MEM which contained, 10% Foetal Bovine Serum (FBS) (PAA, Somerset, UK), 10% penicillin/streptomycin,, 2.5% HEPES, 1% amphotericin and 10% L-glutamine (Sigma Aldrich, UK). The resulting suspension of cells was centrifuged at 1000 rpm for 3 min to form a cell pellet. The cells were then incubated in a 75mL flask in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.'

Statistical analysis

All data were expressed as mean \pm standard deviation of the mean. The t-test was used to evaluate the statistical significance of the measured parameters followed by Shapiro-Wilks test to confirm that the data was normally distributed and therefore the mode of statistical analysis was appropriate.

Results

Pure GG culture beads and nHA/GG composite culture beads with nHA contents of 0.25%, 2.5%, 5% and 25% (w/w) were successfully fabricated using the emulsification method. Examination of the beads using bright-field light microscopy (Figure 1A) showed that the beads were largely spherical with diameter of 415.75 ± 39.28 . The morphology and surface structure of the beads were evaluated using scanning electron microscopy (SEM). The spheres were shown to be uniform and when modified with 25wt% of nHA, particles likely to be the ceramic phase were distributed across the surface of the specimens (Figure 1B). The distribution of particulate material across the surface of the structure seemed to be homogeneous across the surface of the specimens (Figure 1B2).

The response of a population of MC3T3-E1 cells to the GG/nHA particles was investigated using the MTT assay. Preliminary tests after culturing the beads for 7 days demonstrated that the unmodified GG and the GG/nHA composites with nHA concentration up to 2.5% (w/w) did not support cell attachment and proliferation at any time point throughout the study (Figure 2). Modification of the particles with 5wt% nHA enabled cell attachment to the surface of the culture beads and allowed proliferation to occur over a period of seven days (Figure 2).

In order to evaluate whether increasing nHA concentration further increased cell attachment, the culture beads were loaded with up to 25wt% nHA. Increasing the nHA loading of the composite to 25wt% resulted in a significant increase ($p < 0.001$) in cell number by a 2.4 fold on the culture beads over a cultivation period of 7 days (Figure 3i) in comparison with the beads loaded with 5wt% nHA. No significant difference was observed in cell attachment when the nHA loading of the composite was increased. Unmodified GG culture beads did not

facilitate cell attachment or proliferation for the duration of the experiment. The results obtained from the MTT assays were further confirmed through DAPI staining of cell attached culture beads (Figure 3ii), from which, there appeared to be a significant enhancement in cell attachment with the addition of 5%w/w nHA and a further increase with the use of 25%w/w nHA.

As a result the GG/25%nHA was selected for further study using spinner flasks. The feasibility of using these beads in dynamic flask cultures for cell expansion was evaluated and compared with that of conventional monolayer cultures and static bead cultures (Figure 4). Following seeding onto the beads at various conditions, there was a significant ($p < 0.001$) difference between the attachment and proliferation of MC3T3-E1 cells to stirred GG/25%nHA culture beads as compared to static culture beads (Figure 4). In dynamic culture conditions, the cells after the inoculation enter a lag phase from day 0 to 3 days followed by a log phase of cell proliferation (day 4-6) leading to the maximum cell growth at day 7. In contrast the proliferation in static monolayer (control sample) proceeded as might be expected, the cell number increased slowly (day 1-5) and was in stationary phase by day 7 (Figure 4).

The static culture beads followed the same trend as dynamic condition however with a significantly lower rate of cell increase during the cultivation time.

In stirred suspension cultures, the cell adherence and distribution were further observed by SEM. The results show that the cells grew favourably on composite beads. It was shown that the cells appeared to attach to the beads firmly and became flattened on day 3 (Figure 5a, b) and started to form cell bridges with the adjacent beads to form an aggregate of beads (Figure 5c). On day 5, active proliferation of cells was observed over the surface of the beads (Figure

5d). Following the findings presented in Figure 5, further experimentation was undertaken in dynamic conditions.

The functional activity of the MC3T3-E1 pre-osteoblast cells grown on GG/25% nHA beads was examined by measuring ALP activity after culturing for 7 and 14 days as shown in Figure 6. The ALP activity was enhanced at each time-point in cells cultivated on GG/25% nHA beads both in conditioned and un-conditioned culture media without requiring exogenous addition of biochemical factors compared with the cells cultured on TCP in un-conditioned culture media. The ALP activity on control cultures on TCP were only found to be increased when the culture media was supplemented with ascorbic acid, dexamethasone and β -glycerol phosphate as biochemical factors. There was a significant difference ($p < 0.001$) between ALP activity in cells on beads in un-conditioned media and un-conditioned TCP on day 14. (Figure 6d). The increase of ALP activity in cells cultivated on beads in un-conditioned culture media suggest that the fabricated GG/25% nHA nanocomposite in the form of 3D culture beads alone might be sufficient to induce osteogenesis without the presence of biochemical factors.

Mineralised matrix and nodule formation was assessed qualitatively by Alizarin Red staining (Figure 6a,b, and c). Control cultures with no cells showed no alizarin staining (Figure 6a). Cell-beads complexes in osteogenic media showed positive staining indicating the ability of cultured cells on GG/HA beads to deposit mineralized matrix (Figure 6b- top row) as confirmed with ALP activity measurement. Moreover, the culture beads without osteogenic media appeared to be able to induce differentiation and matrix mineralization (Figure 6c- bottom row).

1
2
3 In order to demonstrate the potential of these fabricated beads to stimulate osteogenic
4 differentiation in BMSCs, experiments were undertaken with BMSCs. The ALP activity of
5 cultured BMSCs on GG/25%*n*HA beads were measured after culturing for 7 days (Figure
6 7A). The ALP activity in BMSCs as well as in MC3T3-E1 cells was found to be increased
7 both in osteogenic and non-osteogenic media without the need for addition of biochemical
8 factor compared with control (Non-osteogenic media TCP). There was a significant
9 difference between ALP activity in cells on beads in both conditioned and unconditioned
10 media and in cells in un-conditioned TCP suggesting the osteogenic differentiation of
11 cultivated BMSCs on GG/25%*n*HA beads (Figure 7A). A qualitative Alizarin Red staining
12 assay also indicated the ability of cultured BMSCs on GG/*n*HA beads to induce
13 differentiation and bone nodule formation (Figure 7B).
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Discussion

In this study, GG/nHA composite culture beads were fabricated (Figure 1) and MC3T3-E1 cells were observed to attach, spread, proliferate and form mineralised nodules. It was demonstrated that the incorporation of hydroxyapatite into gellan gum at concentrations of above 5wt% enhanced the ability of gellan hydrogel to support cell attachment and proliferation (Figure 2). The cell attachment and proliferation which was examined with an MTT assay was initiated when the nHA content was increased to 5 wt%. The lack of cellular attachment to unmodified GG was due to the hydrophilic nature of GG, preventing the adsorption of ECM proteins and thereby not enabling cell attachment (Wang et al. 2008; Wang et al. 2010). The incorporation of nanocrystalline HA particles, which have been shown to have a strong adsorptive property for ECM proteins overcome this and support the binding of cells to its surface (Y.-L. Chang et al. 1999; Zhao et al. 2006). Due to this property of nHA, the cell number on the culture beads increased as the nHA content increased (Figure 3).

DAPI staining as well as MTT results qualitatively demonstrated the cell attachment and proliferation of seeded cells on to and within the GG/5%nHA beads and a significant increase of cell number with increasing of HA content up to 25 wt% by day 5 (Figure 3). As a result, the GG/25%nHA was then selected and for further study. The feasibility of using these beads in spinner flask cultures for cell expansion was evaluated and compared to that of conventional monolayer cultures and static beads cultures. The level of cell attachment and proliferation significantly increased in the spinner flask by 2.4 fold in comparison with static culture conditions (Figure 4). The better cell attachment and proliferation in stirred suspension cultures can be explained by the homogeneous culture environment created in the stirred culture and more availability of the entire free surface of beads in suspension culture,

thus the cells had a greater chance of coming into contact with microcarriers (Yu et al. 2004).

Stirred culture systems have been reported to be suitable candidates for the expansion of cells while maintaining the original phenotypic characteristics (Boo et al. 2011). Dynamic culture systems improve the mass transport of oxygen and nutrients to the culture beads that influence a better cell growth in spinner flask (Sikavitsas et al. 2002).

Further analysis of cell-bead complexes by SEM (Figure 5) following culture in spinner flask showed that the cells adhered, proliferated and could form aggregates of cells onto the GG/25% nHA indicating the ability of the fabricated composite beads for cell supporting and aggregate formation which would facilitate their delivery into defect sites (Jos Malda and Frondoza 2006). Recently Tseng et al (2012) found that the altering the culture condition from 2D to 3D microcarrier system is sufficient to induce osteogenesis without the need for osteogenic mediator in culture media through alteration of cytoskeletal tension (Tseng et al. 2012). These two factors alone might be enough to induce osteogenesis and mineralized matrix formation. The elastic modulus of the matrix in which attached cells reside is also an important factor that can have a direct impact on cell differentiation. Cells are known to be responsive to the stiffness of their substrate (Discher et al. 2005). The incorporation of 25wt% into GG matrix enabled the adjustment and increase in elastic modulus by fivefold (Jamshidi, Ma et al. 2012) which might be alone enough to favour the functional activity cultured MC3T3-E1 cells without the need for the presence of osteogenic mediators. The osteogenic potential of these fabricated beads in conditioned and un-conditioned media was also examined with BMSCs cells and the results from ALP activity assay (Figure 7) and calcified matrix formation indicated by positive Alizarin Red histochemical staining in both conditions were also confirmed the suitability and ability of these culture beads for cell delivery in bone tissue regeneration. This characteristic of the GG/nHA culture beads can be

very crucial in simplifying tissue engineering strategies for therapeutic application in regenerative medicine.

Conclusion

In this study, GG/nHA nanocomposites culture beads could be successfully fabricated and applied in spinner flask culture. It was shown that the inclusion of synthetic nHA particles into GG matrix enabled cell attachment to the surface of the composite materials. It was also shown that unmodified GG and the GG/nHA composites with HA concentration up to 2.5% (w/w) did not support cell attachment and proliferation. Cell adhesion and proliferation was significantly improved when the cells cultured on GG/5%nHA and GG/25%nHA beads than when they were cultured on GG beads alone. Furthermore it was found by increasing the content of nHA into GG matrix from 5wt% to 25wt%, the proliferative activity of the MC3T3-E1 cells increased significantly.

Further experiments demonstrated that the dynamic flow environment compared to static conditions enhanced the cell attachment and proliferation of MC-3T3 cells on GG/nHA culture beads. Assessment of ALP activity, on culture beads indicated that ALP activity increased in cells on both conditioned and un-conditioned media compared with TCP in unconditioned media which are only found to be increased when the culture media was supplemented with osteogenic medium. Positive alizarin red staining also indicated that the MC3T3-E1 osteoblast-like cells cultured on 2.5%GG/25%nHA beads have been shown to form aggregates and synthesize mineralized matrix. Importantly such mineralisation occurred without the need for osteogenic mediator in culture demonstrating the remarkable ability of

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2
3 fabricated GG/nHA culture beads to induce osteogenesis and mineralization. These beads
4
5 were also found to be capable to stimulate osteogenic differentiation when BMSCs used in
6
7 the absence of osteogenic media.
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10 11 12 13 14 **Acknowledgments**

15
16 We would like to acknowledge the BBSRC for the provision of a studentship (Parastoo
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18 Jamshidi).
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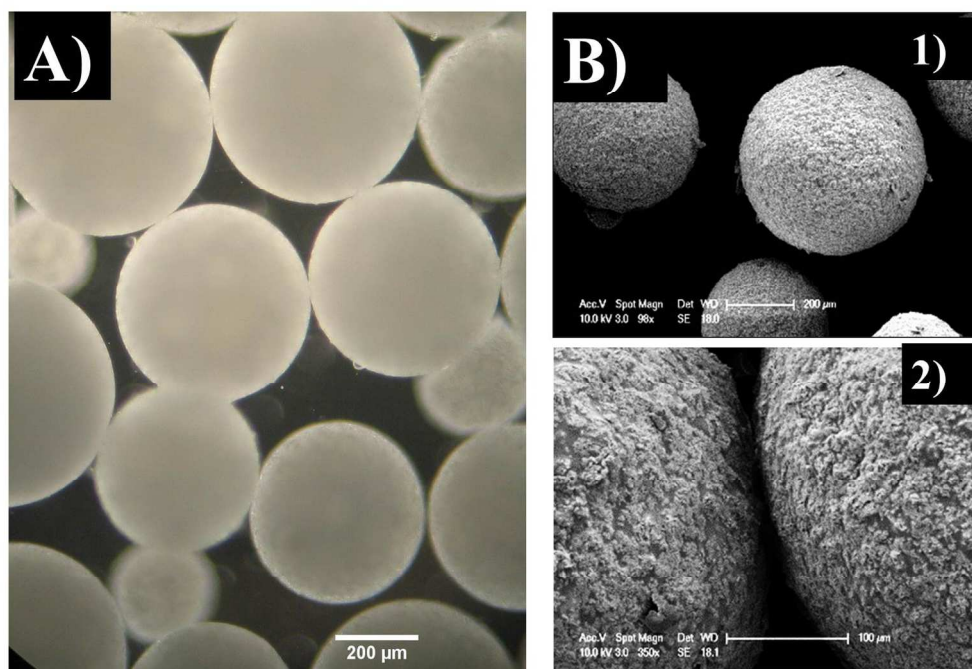
Figure Captions

- **Figure1:** A) An optical micrograph showing the fabricated GG/25wt%nHA beads using the water-in-oil emulsion technique. B) SEM micrograph of GG/25wt%nHA composite beads demonstrating 1) the overall morphology and 2) surface texture of the samples. The beads were of diameter 415.75 ± 39.28 .
- **Figure 2:** MTT assay data showing the influence of nHA addition on the proliferation of attached MC3T3-E1 cells in static condition. The addition of 5%w/w nHA to the gellan was shown to enhance cell attachment significantly ($*p < 0.001$) to the culture surfaces.
- **Figure 3:** i) Comparison of cell proliferation in static condition on GG/5%nHA and GG/25%nHA culture beads determined by MTT assay on MC3T3-E1 cells grown on the. GG beads didn't facilitate cell attachment and cell growth at any time-point throughout the study. The number of cells on GG/25%nHA beads significantly increased over the duration of the study ($p < 0.001$) than that of GG/5%nHA beads over the cultivation period (Data points represent mean values of $n = 9$ specimens \pm standard deviation) and ii) Cell attachment visualisation using DAPI staining; a) and d) cell- free beads (control samples, with 25%w/w), b and e) cell-GG/5%nHA beads complexes, c and f) cell-GG/25%nHA beads complexes after culturing for 5 days. The images confirms significant increase in cell number and proliferation of MC3T3-E1 cells on GG/25%nHA beads compared to that of GG/5%nHA beads. Scale bar=100 μ m.

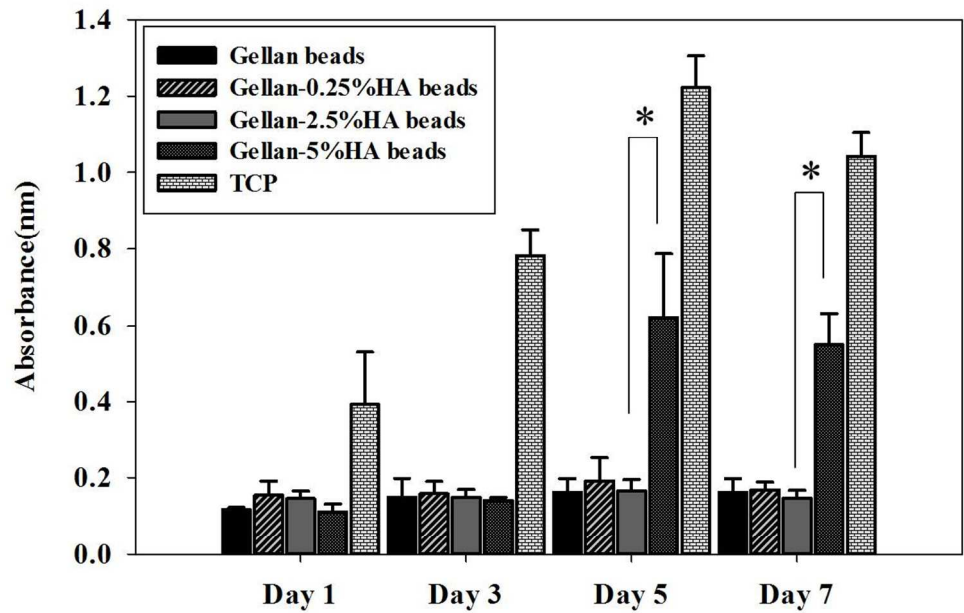
- **Figure 4:** Comparison of cell proliferation on GG/25%*n*HA beads in static and dynamic conditions (spinner flask) with a control monolayer culture. There was a significant difference ($p < 0.001$) between the attachment and proliferation of MC3T3-E1 cells to the dynamic GG/25%*n*HA beads as compared to monolayer culture and static GG/25%*n*HA beads were seen at all-time points. Data points represent mean values of $n = 9$ specimens \pm standard deviation.
- **Figure 5:** SEM micrographs of the MC3T3-E1 cells grown on the surface of 25wt% *n*HA/GG culture beads; a, b, c) day 3, and d) day 5.
- **Figure 6:** Alizarin Red Staining of MC3T3-E1 cells (dynamic condition) indicating of mineralized matrix synthesis. a) Control-beads with no cells, b) cell-beads complexes in osteogenic media for 3 days, c) cell-beads complexes in osteogenic media for 5 days. Top row is cell-beads complexes in osteogenic media and bottom row is in non-osteogenic media (Scale bars=100 μ m) and d) Comparison of Alkaline phosphatase (ALP) activity on the GG/25%*n*HA beads after culturing for up to 7 and 14 days in osteogenic and non-osteogenic media. TCP was used as a control. ALP activity was normalised to cell number. The difference between ALP activity in cells on beads in un-conditioned media and in un-conditioned TCP at day 14 was significant $*p < 0.001$.
- **Figure 7:** A) Comparison of the ALP activity for the BMSCs cultured on GG/*n*HA beads (dynamic condition) in osteogenic media (OS-Beads), non-osteogenic media (Non OS-Beads) and Osteogenic and non-osteogenic tissue culture plastic (OS TCP and Non OS-TCP). Cells cultured in OS-beads and Non OS-Beads were significantly higher than that of the cells in NonOS- TCP. $*p < 0.05$ when comparing OS-beads and

Non OS- TCP. # $p<0.001$ when comparing Non OST-beads and Non OS-TCP. B)
Alizarin red staining of BMSCs in 1) osteogenic media and 2) non-osteogenic media
indicating the ability of cells to induce matrix mineralization. Results are displayed as
mean of $n = 9$ specimens \pm standard deviation. Scale bar = $100\mu\text{m}$

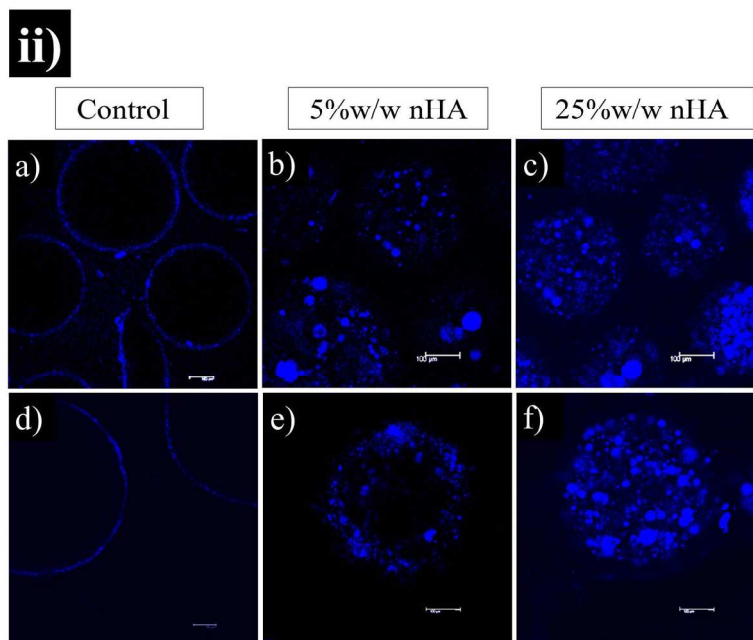
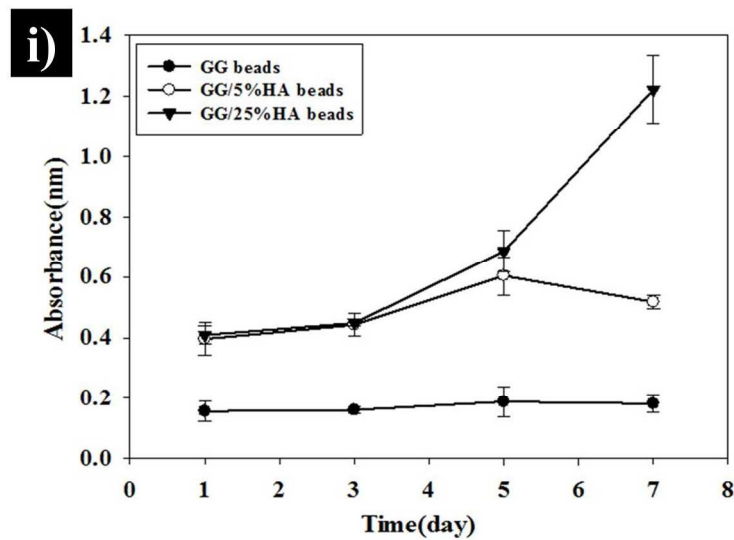
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• Figure1: A) An optical micrograph showing the fabricated GG/25wt%nHA beads using the water-in-oil emulsion technique. B) SEM micrograph of GG/25wt%nHA composite beads demonstrating 1) the overall morphology and 2) surface texture of the samples. The beads were of diameter 415.75 ± 39.28 . 289x196mm (150 x 150 DPI)

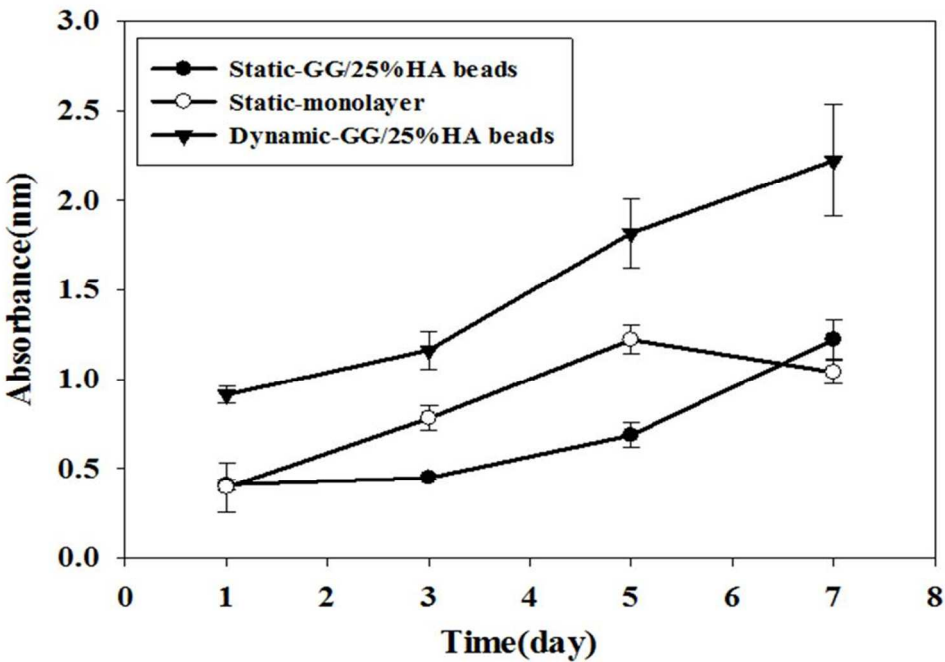


• Figure 2: MTT assay data showing the influence of nHA addition on the proliferation of attached MC3T3-E1 cells in static condition. The addition of 5%w/w nHA to the gellan was shown to enhance cell attachment significantly (*p<0.001) to the culture surfaces.
210x146mm (150 x 150 DPI)



• Figure 3: i) Comparison of cell proliferation in static condition on GG/5%nHA and GG/25%nHA culture beads determined by MTT assay on MC3T3-E1 cells grown on the. GG beads didn't facilitate cell attachment and cell growth at any time-point throughout the study. The number of cells on GG/25%nHA beads significantly increased over the duration of the study ($p < 0.001$) than that of GG/5%nHA beads over the cultivation period (Data points represent mean values of $n = 9$ specimens \pm standard deviation) and ii) Cell attachment visualisation using DAPI staining; a) and d) cell-free beads (control samples, with 25%w/w), b) and e) cell-GG/5%nHA beads complexes, c) and f) cell-GG/25%nHA beads complexes after culturing for 5 days. The images confirm significant increase in cell number and proliferation of MC3T3-E1 cells on GG/25%nHA beads compared to that of GG/5%nHA beads. Scale bar=100 μ m.

332x504mm (150 x 150 DPI)



• Figure 4: Comparison of cell proliferation on GG/25%HA beads in static and dynamic conditions (spinner flask) with a control monolayer culture. There was a significant difference ($p < 0.001$) between the attachment and proliferation of MC3T3-E1 cells to the dynamic GG/25%HA beads as compared to monolayer culture and static GG/25%HA beads were seen at all-time points. Data points represent mean values of $n = 9$ specimens \pm standard deviation.
186x140mm (150 x 138 DPI)

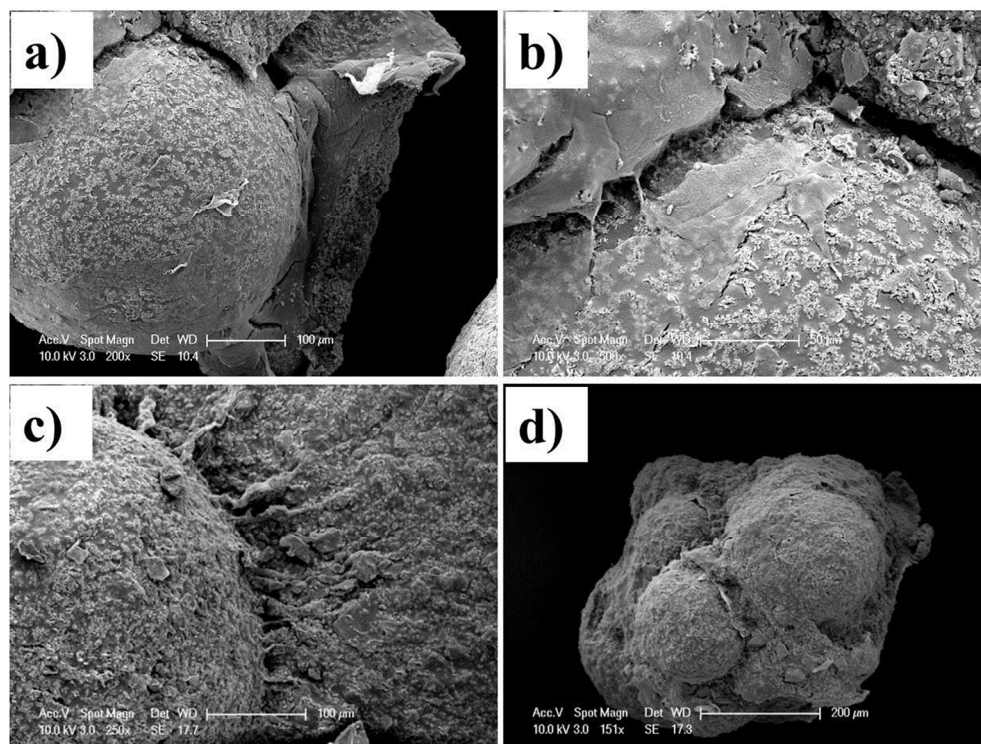
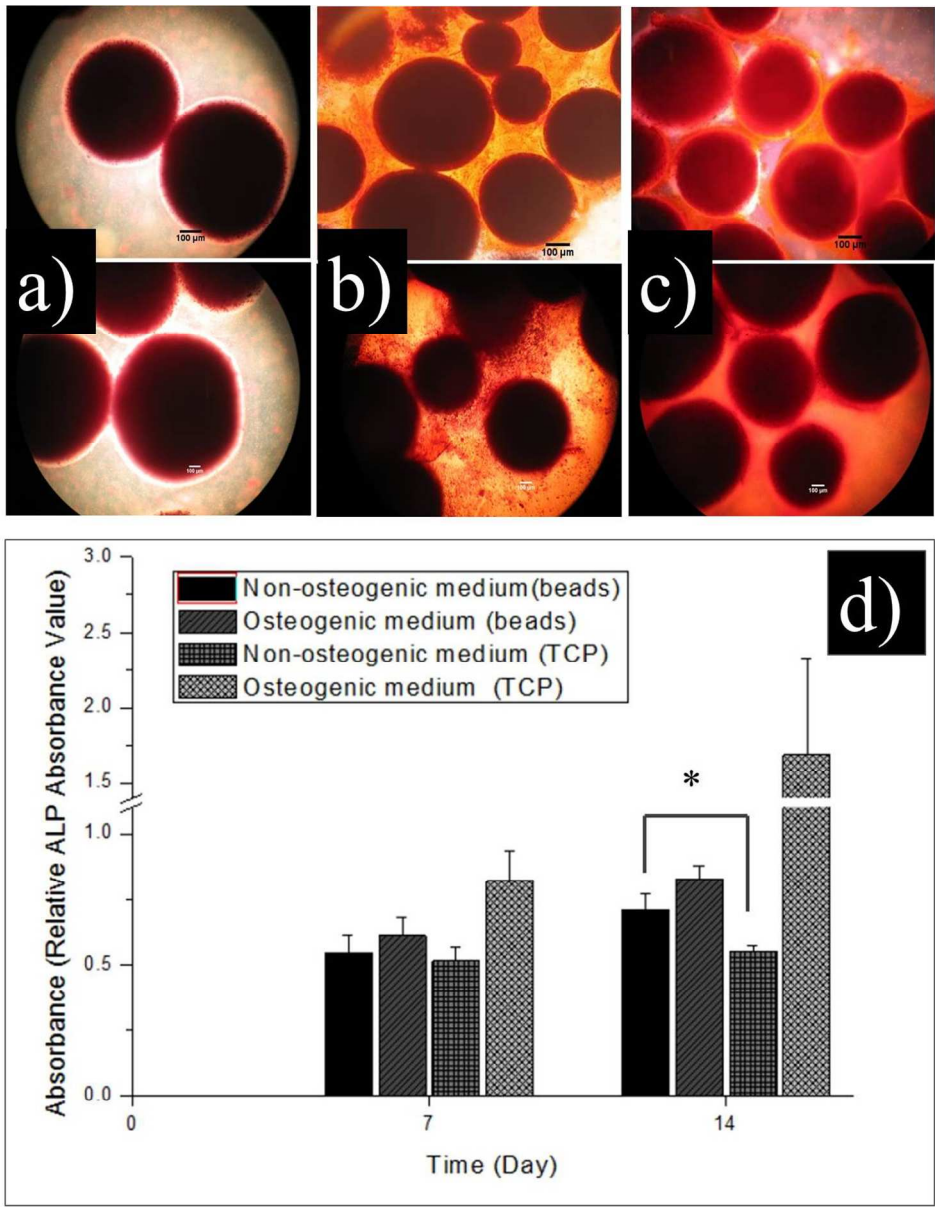
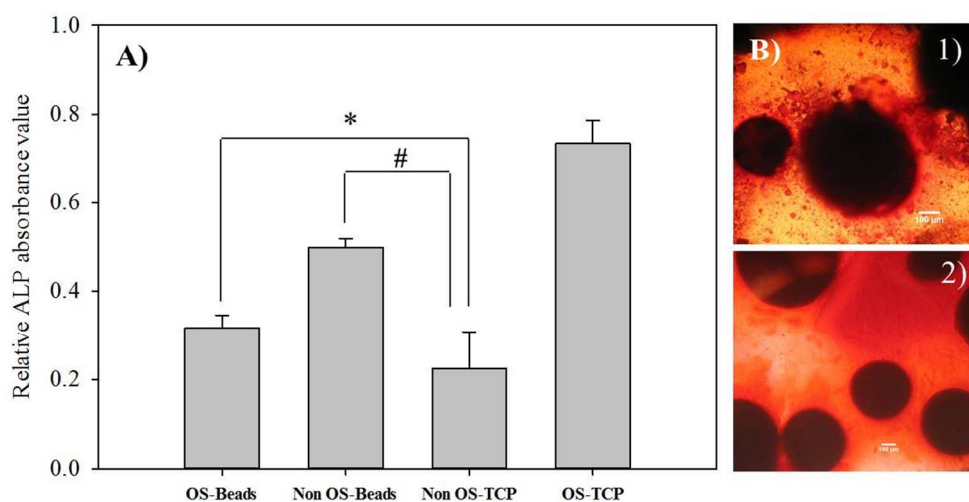


Figure 5: SEM micrographs of the MC3T3-E1 cells grown on the surface of 25wt% nHA/GG culture beads; a, b, c) day 3, and d) day 5.
214x160mm (150 x 150 DPI)



• Figure 6: Alizarin Red Staining of MC3T3-E1 cells (dynamic condition) indicating of mineralized matrix synthesis. a) Control-beads with no cells, b) cell-beads complexes in osteogenic media for 3 days, c) cell-beads complexes in osteogenic media for 5 days. Top row is cell-beads complexes in osteogenic media and bottom row is in non-osteogenic media (Scale bars=100μm) and d) Comparison of Alkaline phosphatase (ALP) activity on the GG/25% nHA beads after culturing for up to 7 and 14 days in osteogenic and non-osteogenic media. TCP was used as a control. ALP activity was normalised to cell number. The difference between ALP activity in cells on beads in un-conditioned media and in un-conditioned TCP at day 14 was significant *p<0.001.

241x295mm (150 x 150 DPI)



• Figure 7: A) Comparison of the ALP activity for the BMSCs cultured on GG/nHA beads (dynamic condition) in osteogenic media (OS-Beads), non-osteogenic media (Non OS-Beads) and Osteogenic and non-osteogenic tissue culture plastic (OS TCP and Non OS-TCP). Cells cultured in OS-beads and Non OS-Beads were significantly higher than that of the cells in NonOS- TCP. * $p < 0.05$ when comparing OS-beads and Non OS-TCP. # $p < 0.001$ when comparing Non OST-beads and Non OS-TCP. B) Alizarin red staining of BMSCs in 1) osteogenic media and 2) non-osteogenic media indicating the ability of cells to induce matrix mineralization. Results are displayed as mean of $n = 9$ specimens \pm standard deviation. Scale bar = 100 μm 202x113mm (150 x 150 DPI)